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MECHANISMS OF RESISTANCE

Functional Characterization of the DNA Gyrases in Fluoroquinolone-Resistant Mutants of *Francisella novicida*

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ABSTRACT Fluoroquinolone (FQ) resistance is a major health concern in the treatment of tularemia. Because DNA gyrase has been described as the main target of these compounds, our aim was to clarify the contributions of both GyrA and GyrB mutations found in *Francisella novicida* clones highly resistant to FQs. Wild-type and mutated GyrA and GyrB subunits were overexpressed so that the *in vitro* FQ sensitivity of functional reconstituted complexes could be evaluated. The data obtained were compared to the MICs of FQs against bacterial clones harboring the same mutations and were further validated through complementation experiments and structural modeling. Whole-genome sequencing of highly FQ-resistant lineages was also done. Supercoiling and DNA cleavage assays demonstrated that GyrA D87 is a hot spot FQ resistance target in *F. novicida* and pointed out the role of the GyrA P43H substitution in resistance acquisition. An unusual feature of FQ resistance acquisition in *F. novicida* is that the first-step mutation occurs in GyrB, with direct or indirect consequences for FQ sensitivity. Insertion of P466 into GyrB leads to a 50% inhibitory concentration (IC_{50}) comparable to that observed for a mutant gyrase carrying the GyrA D87Y substitution, while the D487E-ΔK488 mutation, while not active on its own, contributes to the high level of resistance that occurs following acquisition of the GyrA D87G substitution in double GyrA/GyrB mutants. The involvement of other putative targets is discussed, including that of a ParE mutation that was found to arise in the very late stage of antibiotic exposure. This study provides the first characterization of the molecular mechanisms responsible for FQ resistance in *Francisella*.

KEYWORDS DNA gyrase, fluoroquinolones, *Francisella*

Francisella tularensis is a Gram-negative, facultatively intracellular bacterium responsible for the zoonosis tularemia (1). Classically, the following two subspecies cause most human infections: *F. tularensis* subsp. *tularensis* (type A) in North America and *F. tularensis* subsp. *holarctica* (type B) throughout the Northern Hemisphere. Humans are contaminated through direct contact with infected animals (especially hares), arthropod bites (mainly from ticks), ingestion of contaminated food or water, and contact with contaminated environments. Depending on the portal of entry of bacteria, the disease may manifest in six clinical forms: the ulceroglandular and glandular forms, which are regional lymphadenopathies with and without a visible skin inoculation lesion, respectively; the oropharyngeal form, a pharyngitis with cervical lymphadenopathy; the oculoglandular form, a conjunctivitis with a pretragal or cervical lymphade-

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nopathy; the pneumonic form, acquired after inhalation of a contaminated aerosol or through hematogenous spread of bacteria; and the typhoidal form, combining high fever and neurological symptoms that may mimic those of typhoid. Among these various clinical forms, respiratory tularemia has attracted the most attention because it may cause up to 30% mortality in humans if left untreated (2, 3). This highly infectious aerosolizable pathogen is considered a class A bioterrorism agent by the Centers for Disease Control and Prevention. However, despite its potential use as a biological weapon, no safe and potent vaccine is currently available (2, 3). A few antibiotic classes are effective for treating tularemia, including aminoglycosides, tetracyclines, and fluoroquinolones (FQs) (4). Microbiological and clinical data show that ciprofloxacin, and possibly other FQs, is the best alternative for oral therapy of tularemia in patients with clinical manifestations of mild to moderate severity, but failures and relapses are frequent (5, 6).

Quinolones are one of the most commonly prescribed classes of antibacterial agents in the world (7). These drugs inhibit DNA replication through interaction with complexes composed of DNA and either of the two target enzymes, DNA gyrase or topoisomerase IV, which belong to the type IIA topoisomerases (8). As in most Gram-negative bacteria, the primary target for FQs in *Francisella* strains is thought to be the DNA gyrase (9–11), which functions as an A_2B_2 heterotetrameric complex able to catalyze negative supercoiling of the bacterial circular chromosome (12). Resistance to FQs can result from single point mutations in GyrA and GyrB, leading to conformational changes of the whole complex, which in turn impair antibiotic-target interactions. Such mutations are described to be restricted mainly to discrete regions of the so-called quinolone resistance-determining regions (QRDRs). Another major mechanism of resistance relies on mutations affecting the bacterial entry/efflux systems, resulting in reduced quinolone accumulation in bacterial cells. Most of these efflux pumps are encoded on the chromosome, but quinolone-specific (QepA) as well as multidrug (OqxAB) efflux pumps were also found to be encoded by bacterial plasmids. Other plasmid-mediated quinolone resistance factors include the *qnr* genes, encoding pentapeptide repeat proteins that act as DNA mimics and protect the type II topoisomerases, and an allele of the aminoglycoside acetyltransferase-encoding gene (*aac*) that is able to decrease drug activity through acetylation (7, 13, 14).

Because of the widespread use (and overuse) of these drugs, the increased prevalence of quinolone-resistant strains is a growing concern for several bacterial species (15). To date, no resistant strain of *F. tularensis* has been isolated from patients (16), but successful culture of this fastidious pathogen is achieved in only 10% of tularemia cases (5). *In vitro*, the C248T mutation (encoding a T83I substitution) was found in the GyrA QRDR of a quinolone-resistant *F. tularensis* subsp. *holarctica* strain (URFtCIPR isolate) (9). This mutation was also observed in an *F. tularensis* SchuS4 strain isolated after *in vitro* exposure to increasing amounts of ciprofloxacin and was accompanied by the G259T (D87Y) GyrA QRDR substitution (10).

An evolution experiment was conducted in our lab by exposure of several *Francisella* sp. strains with attenuated virulence for humans to increasing concentrations of FQs (11). This approach, considered a powerful tool for investigating the acquisition dynamics of drug resistance (17, 18), led to the emergence of high-level resistant mutants on which phenotypic and genotypic analyses were carried out (11). Besides mutations in the QRDR-A and QRDR-B hot spot regions, novel substitutions or deletions were identified in both the *Francisella* GyrA and GyrB subunits. An elegant study by Jaing et al. (19) recently reported genome-wide mutations associated with ciprofloxacin-resistant *F. tularensis* LVS isolated *in vitro* and confirmed the GyrA and GyrB mutations described by Sutera et al. (11). However, while a structural model of DNA gyrase was built to achieve a better understanding of how mutations affect FQ resistance (19), their functional role was not investigated.

Our aim in the present study was to clarify the functional consequences of GyrA and GyrB mutations on FQ sensitivity in four independent lineages of *F. novicida* highly resistant to such antibiotics. While this *Francisella* species is not pathogenic to humans,

TABLE 1 Characteristics of the *F. novicida* lineages used in this study[a]

Lineage	MIC (mg/liter)	Amino acid mutation(s)		Reference or strain no.
		GyrA	GyrB	
Fno_WT	0.064	No mutation	No mutation	CIP56.12
Fno1	128	D87Y	+P466	11
Fno2	128	D87G	D487E, ΔK488	11
Fno3	32	ΔE524, ΔS525	No mutation	11
Fno4	64	P43H	No mutation	11

[a]The bacteria used in this study included the FQ-sensitive parental strain *F. novicida* U112 (Fno_WT) and four lineages (Fno1 to Fno4) that acquired high FQ resistance levels associated with mutations in GyrA and GyrB after 14 subcultures in the presence of antibiotics (11). The ciprofloxacin MICs were measured on the bacterial clones selected for whole-genome sequencing at the final stage of the evolution procedure, using Mueller-Hinton 2 broth supplemented with 2% PolyViteX as described previously (11).

it is a widely used model for studying *F. tularensis* infections (20). This is the first study to describe the DNA supercoiling activity and FQ-induced DNA cleavage activity of *Francisella* proteins, using these functional characteristics as efficient ways to establish relationships between DNA gyrase mutations and FQ resistance (21).

RESULTS

Expression and purification of recombinant *F. novicida* DNA gyrases. To assess the relationships between resistance levels and mutations in GyrA and GyrB, we cloned and expressed the wild-type and mutated proteins listed in Table 1. High yields of recombinant His-tagged and soluble recombinant GyrB proteins were obtained with only a few changes of the protocol described by Tari et al. (22). In contrast, several attempts were required to optimize purification of the GyrA subunits, including variations in temperature, induction times, salt concentrations, and addition of detergents. As judged by SDS-PAGE analysis (see Fig. S1 in the supplemental material), the purity of all GyrA and GyrB constructs was >90%.

DNA supercoiling activity of *F. novicida* DNA gyrases. The wild-type DNA gyrase of *F. novicida* was reconstituted *in vitro* by use of equimolar amounts of the wild-type GyrA and GyrB proteins and examined for the capacity to convert relaxed pBR322 to its supercoiled form. As shown in Fig. 1, performing the assay with 35 mM Tris-HCl, pH 7.5, 24 mM KCl, 2 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), 1.8 mM spermidine, and various concentrations of $MgCl_2$ and ATP demonstrated that the reconstituted complex could catalyze a magnesium-dependent supercoiling reaction, although the effect was not optimal. The catalytic activity of *F. novicida* DNA gyrase was significantly improved by the addition of 100 mM potassium glutamate (KGlu) and required $MgCl_2$ and ATP at optimum concentrations of 2 and 1 mM, respectively.

Respective contributions of GyrA and GyrB mutations to FQ resistance. To assess the respective roles of the GyrA and GyrB mutations in ciprofloxacin resistance, the inhibitory effect of FQs was first tested against the supercoiling activity of DNA gyrase complexes corresponding to the wild-type sensitive strain or complexes reconstituted using a recombinant mutated target in combination with its complementary

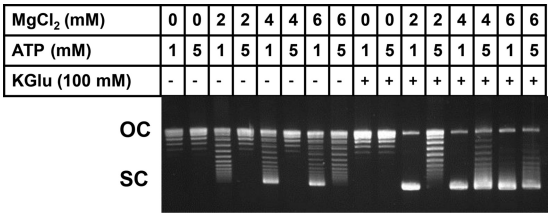

FIG 1 Supercoiling activity of reconstituted *F. novicida* DNA gyrase complex. Recombinant GyrA and GyrB subunits from *F. novicida* (250 ng of each) were assayed for DNA supercoiling activity for 30 min at 37°C, with 0.15 μg relaxed pBR322 as the substrate and in the presence of different concentrations of $MgCl_2$, ATP, and KGlu, as indicated. The DNA product was analyzed in a 0.8% agarose gel. OC and SC, open circular and supercoiled forms of pBR322, respectively.

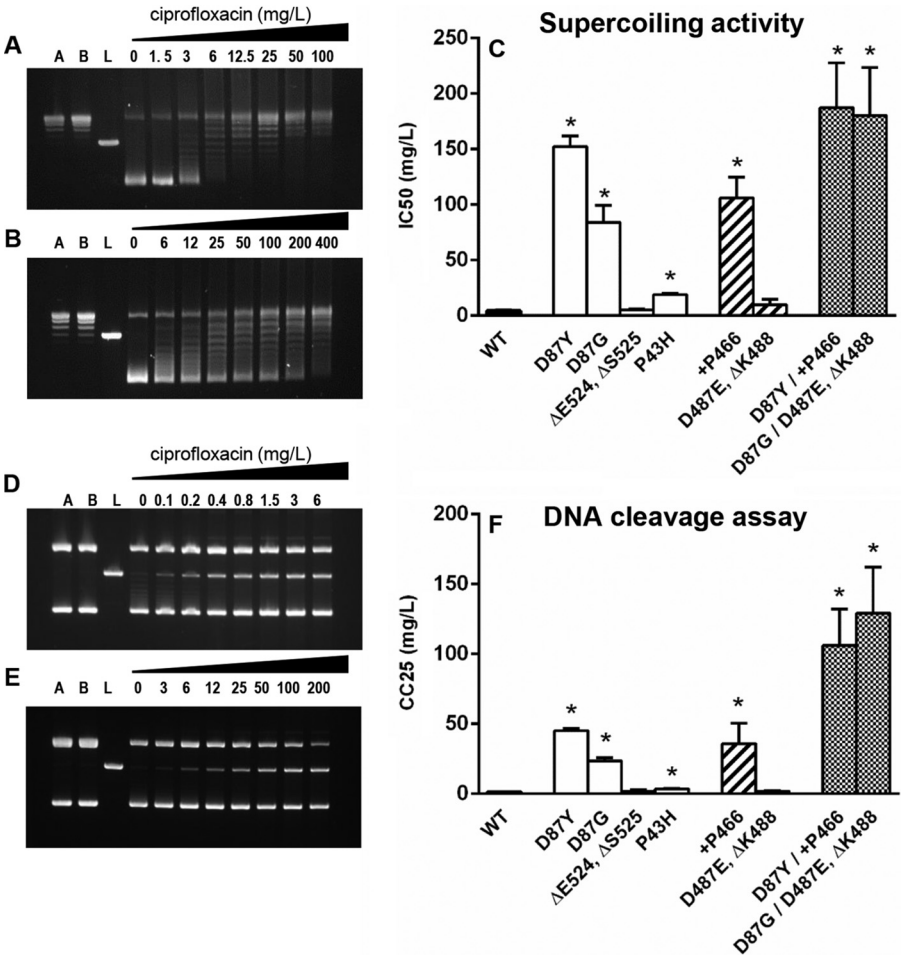

FIG 2 Ciprofloxacin inhibits DNA supercoiling and promotes DNA cleavage induced by wild-type and mutated DNA gyrases from *F. novicida*. The inhibitory activity of increasing concentrations of ciprofloxacin was measured for 30 min at 37°C against DNA supercoiling activity induced by DNA gyrase complexes reconstituted with recombinant *F. novicida* wild-type GyrA and GyrB (A) or the mutated GyrA (D87Y) and GyrB (+P466) proteins found in the resistant Fno1 lineage (B). Lanes A, relaxed pBR322 and GyrA (2 U); lanes B, relaxed pBR322 and GyrB (2 U); lanes L, pBR322 linearized by BamHI digestion; lanes 4 to 11, supercoiling activity obtained in the presence of increasing concentrations of ciprofloxacin in comparison to that with the positive control (2 U of each subunit) without an antibiotic. The gels are representative of at least three distinct experiments. (C) Ciprofloxacin sensitivity, expressed as the IC_{50}, was measured on DNA gyrase complexes sharing mutations in GyrA (white columns), GyrB (hatched columns), or both (dotted columns). (D to F) The same experiments as those described above, but using supercoiled pBR322 incubated for 1 h at 25°C in the presence of fixed amounts of recombinant proteins (125 nM) and increasing concentrations of ciprofloxacin. Fragmented DNA was detected following addition of proteinase K and SDS. Data represent the means ± SEM for three to nine distinct experiments conducted with two different batches of recombinant proteins. *, $P < 0.05$.

wild-type GyrA or GyrB subunit. Representative inhibition profiles are shown in Fig. 2A and B. For each condition, we verified that neither the GyrA nor GyrB subunit alone induced DNA supercoiling that could have resulted from a putative contamination of the recombinant proteins with the *Escherichia coli* enzymes (wells 1 and 2). Given that similar but not strictly identical patterns were obtained for experiments conducted with different batches of proteins, the data were expressed as means ± standard errors of the means (SEM) for the ciprofloxacin concentrations required to inhibit 50% of DNA supercoiling activity (IC_{50}) in different assays. As illustrated in Fig. 2C, the substitutions at position 87 in QRDR-A generated a high level of FQ resistance, with an IC_{50} approximately twice as high when the aspartic acid residue was exchanged for a tyrosine as that when the aspartic acid was exchanged for a glycine (152.3 ± 9.2 mg/liter [$n = 4$] for the D87Y mutant versus 83.82 ± 15.2 mg/liter [$n = 6$] for the D87G

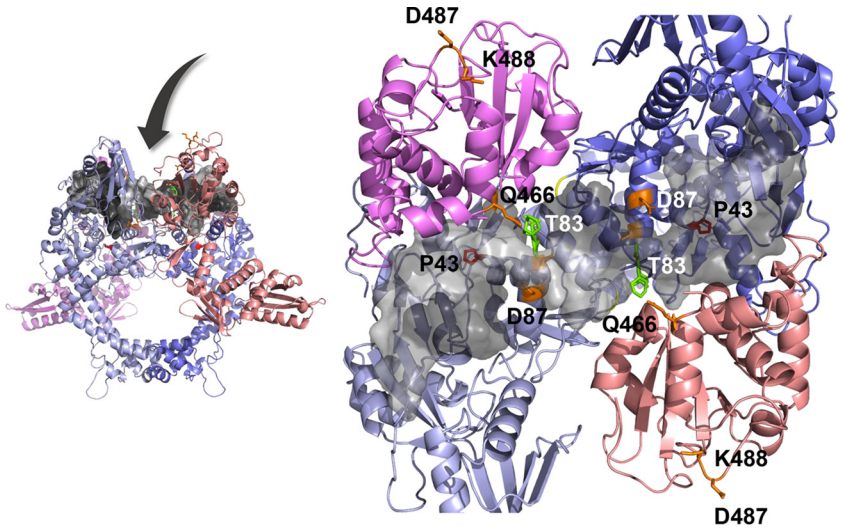

FIG 3 Three-dimensional structure modeling of the *F. novicida* QRDR. (Left) Front view of the complex, with the Toprim domains represented in magenta and pink, the breakage reunion domains in blue, and DNA in transparent gray. The black arrow indicates the location of the QBP of the catalytic core. (Right) Top-view zoom of the QBP. The catalytic Y122 residues are colored yellow, and residues at positions critical for FQ resistance are indicated.

mutant) ($P < 0.05$). To a lesser extent, but significantly, the P43H substitution in the GyrA N-terminal domain resulted in a ciprofloxacin sensitivity that was four times lower (18.9 ± 1.07 mg/liter [$n = 6$]) than that of the wild-type complex (4.11 ± 0.91 mg/liter [$n = 5$]). The three-dimensional (3D) structure of the *F. novicida* quinolone-binding pocket (QBP) together with FQs and DNA that was generated through homology modeling (Fig. 3) shows that this residue is located close to the catalytic Y122 residues where the DNA is bound and close to the D87 residues that interact with FQ. From this model, it can be assumed that the P-to-H substitution potentially affects the inhibitory effect of the drugs. Finally, the ΔE524 and ΔS525 deletions found in the C terminus of GyrA did not affect drug activity (4.98 ± 0.65 mg/liter [$n = 3$]).

Supercoiling data also evidenced that the QRDR-B insertion (+P466) led to a high resistance level (105.09 ± 18.86 mg/liter [$n = 6$]), in contrast to the D487E-ΔK488 mutation found in the C-terminal domain of the GyrB subunit, which failed to significantly affect FQ sensitivity (9.6 ± 1.07 mg/liter [$n = 5$]). The capacity of ciprofloxacin to inhibit DNA supercoiling activity was then evaluated against reconstituted DNA gyrase complexes corresponding to the high-level resistant clones isolated after the final (14th) passage of the Fno1 and Fno2 lineages sharing GyrA/GyrB double mutations (Fig. 2C). The IC_{50} measured with the reconstituted Fno1 complex, which possesses mutations in both QRDR-A (D87Y) and QRDR-B (+P466), was 186.9 ± 40.69 mg/liter ($n = 6$). This value was not significantly different from the value obtained with the reconstituted Fno2 complex (181.1 ± 43.09 mg/liter [$n = 9$]), which has a QRDR-A substitution (D87G) accompanied by a GyrB mutation outside the QRDR (D487E-ΔK488). A strictly similar pattern was obtained using moxifloxacin (not shown). Overall, these results are well correlated with the data obtained from the ciprofloxacin-mediated DNA cleavage assays (Fig. 2D to F; Table S3).

Stepwise appearance of GyrA and GyrB mutations. QRDR-A and the mutated GyrB regions were sequenced from the *F. novicida* Fno1 and Fno2 initial lineages as well as from resistant isolates saved at the different passages during the course of antibiotic exposure (11). For each passage of interest, six clones were analyzed and showed similar results. In both cases, and in contrast to what was expected, the GyrB mutation occurred first. In Fno1, the CTC nucleotide insertion leading to the appearance of an additional proline residue (+P466) in the GyrB subunit was detected as early as the second passage. The G-to-T substitution responsible for the D-to-Y change at position

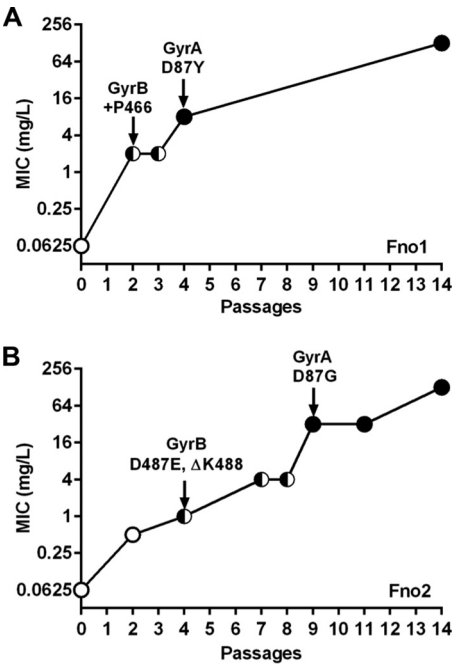

FIG 4 Stepwise appearance of GyrA/GyrB mutations and FQ resistance. The QRDR-A and mutated GyrB regions from initial lineages and resistant clones of *F. novicida* saved at different passages during the course of antibiotic exposure were sequenced using specific primers (Table S1) to check for the appearance of mutations, which are indicated by black symbols and arrows on the graph. Susceptibility to ciprofloxacin was also evaluated before and after acquisition of mutations; MICs are presented as MIC modal values. (A) Lineage Fno1; (B) lineage Fno2.

87 of QRDR-A arose later (passage 4). Similarly, in Fno2, the three-base deletion (TAA; positions 1460 to 1462) accounting for the D487E-ΔK488 mutation in GyrB was found at passage 4, while the A260G point mutation accounting for the GyrA QRDR D87G substitution occurred at passage 9. To evaluate the precise impacts of the detected mutations on ciprofloxacin resistance, MICs were then measured on the corresponding bacterial clones both before and after each mutation occurred. The chronological appearance of GyrA and GyrB mutations and the evolution of the ciprofloxacin MICs are summarized in Fig. 4. The shifts in the MIC observed upon acquisition of a QRDR-A substitution in bacterial isolates sharing a GyrB mutation (×4 in Fno1 and ×32 in Fno2) were within the same range of magnitude as the IC_{50} shifts obtained with the recombinant DNA gyrase complexes carrying either single GyrB or double GyrA/GyrB mutations (see above). This result reinforces the contribution of DNA gyrase mutations to the FQ resistance of evolved bacterial clones. Moreover, the important shift in resistance observed upon acquisition of the QRDR-A mutation in Fno2, together with the moderate impact of the FQ resistance-conferring D87G mutation, suggests that the D487E-ΔK488 mutation found in GyrB, which fails to significantly affect the resistance phenotype on its own, indirectly contributes to an increased FQ resistance level.

Complementation study confirms the contribution of the GyrB D487E-ΔK488 mutation to FQ resistance. Complementation experiments were conducted on the *F. novicida* Fno2 single mutant (passage 4; QRDR-B D487E-ΔK488) or double mutant (passage 11; QRDR-B D487E-ΔK488 plus QRDR-A D87G), using a pFNLTP6 plasmid carrying the gene encoding either wild-type or mutated GyrB. Whichever plasmid was used, the MIC value obtained upon complementation of the *F. novicida* Fno2 clone isolated at passage 4, i.e., before acquisition of the GyrA mutation, remained unchanged. In contrast, we observed that the ciprofloxacin susceptibility of the clone isolated at passage 11 was partially restored upon complementation with wild-type GyrB, with this transformation conferring an approximately 2- to 3-fold decrease in the MIC relative to that for the same mutant transformed with the mutated protein (64 mg/liter).

TABLE 2 Single nucleotide polymorphisms and indels identified by whole-genome sequencing of the highly resistant evolved lineages

	Mutation(s)		Other mutations identified in the genomes of sequenced mutants				
Lineage	GyrA	GyrB	Gene no.[a]	Gene name	Description	Type of mutation	Consequence
Fno1	D87Y	+P466	FTN_0480	*fevR*	*Francisella* effector of virulence regulation	Point mutation in the promoter	Unknown
			FTN_1418	*manC*	Mannose-1-phosphate guanylyltransferase	Insertion	Stop codon 38
			FTN_1510	*secB2*	Preprotein translocase, subunit B	Deletion	Stop codon 89
			FTN_1652	*atpB*	ATP synthase	Deletion	Stop codon 10
Fno2	D87G	D487E, ΔK488	FTN_0402	*aroC*	Chorismate synthase	Deletion	Stop codon 140
			FTN_0480	*fevR*	*Francisella* effector of virulence regulation	Point mutation in the promoter	Unknown
			FTN_1083		Hypothetical protein	Point mutation	D128N mutation
			FTN_1421	*wbtH*	Glutamine amidotransferase/asparagine synthase	Deletion	Stop codon 313
			FTN_1550	*parE*	Topoisomerase IV, subunit B	Point mutation	P742S mutation
			FTN_1610		RND efflux transporter, AcrB/AcrD/AcrF family	Point mutation	Y320H mutation
Fno3	ΔE524, ΔS525		FTN_0444*	*fupA*	Fer utilization protein	Deletion	Stop codon 39
			FTN_0480	*fevR*	*Francisella* effector of virulence regulation	Point mutation in the promoter	Unknown
			FTN_0913	*rpoD*	RNA polymerase sigma-70 factor	Deletion	Deletion D52
			FTN_1029		Isoprenoid biosynthesis protein with amidotransferase-like domain	Insertion	Stop codon 43
			FTN_1115	*pilB*	Type IV pilus ATPase	Insertion	Stop codon 180
			FTN_1430*	*wbtQ*	Aminotransferase	Deletion	Stop codon 145
Fno4	P43H		FTN_0070	*pilE*	Type IV pili, pilus assembly protein	Deletion	Stop codon 117
			FTN_0480	*fevR*	*Francisella* effector of virulence regulation	Point mutation in the promoter	Unknown
			FTN_0504		Lysine decarboxylase	Insertion	Stop codon 612
			FTN_1471	*pcs*	(CDP-alcohol) phosphatidyltransferase	Deletion	Stop codon 92
			FTN_1609*		MexH family multidrug efflux RND transporter periplasmic adaptor subunit	Deletion	Deletion of Y101

[a]*, gene found to contain mutations in ciprofloxacin-resistant *F. tularensis* isolates (19).

Genome sequencing of *F. novicida* clones highly resistant to FQs. As shown above, the GyrA deletion found in the Fno3 lineage was not associated with FQ resistance; concerning Fno4, the inhibitory effect of ciprofloxacin on DNA supercoiling *in vitro* (Fig. 2C) did not correlate with the high MIC measured for the final bacterial isolates (Table 1). In addition, sequencing of *gyrA* and *gyrB* in the Fno1 and Fno2 lineages at different passages clearly showed that the MIC continued to increase after the appearance of mutations in these FQ target genes. Together, these data suggest that mutational events may occur in other genes. The genomic analysis of the four lineages highly resistant to FQs (passage 14) is summarized in Table 2. An interesting feature found in the Fno2 lineage was a mutation of the topoisomerase IV gene encoding ParE. However, sequencing data revealed that this mutation occurred at the final stage of antibiotic exposure (passage 14), as previously reported for the *F. tularensis* lineage (11). These data revealed that, besides changes in the DNA gyrases, another characteristic shared between all these isolates was a mutation within the promoter of the FTN_0480 gene. Other mutations detected were specific for each clone. Their putative impacts on FQ resistance are discussed below.

DISCUSSION

In the present study, we successfully produced the recombinant GyrA and GyrB subunits found in FQ-resistant and -susceptible lineages of *F. novicida*. All affinity-purified proteins were enzymatically active, as shown by the capacity of reconstituted complexes to promote supercoiling of relaxed pBR322. This specific *in vitro* DNA supercoiling assay has not been reported before for *Francisella* proteins and allowed us to clarify the roles of the different mutations in the FQ susceptibility of these bacterial pathogens.

One of the main features of *F. novicida* lineages exposed to FQs is a substitution affecting the D87 residue, located in QRDR-A, that has been associated with acquired quinolone resistance in several bacterial species, including *E. coli* and *Mycobacterium tuberculosis* (23) (corresponding residue D94 [see Fig. S2 in the supplemental material]). The work presented here clearly demonstrates that this residue is also a hot spot quinolone resistance target in *Francisella* strains. Its replacement by glycine or tyrosine indeed confers high-level resistance on mutated recombinant DNA gyrases from *F. novicida*, a result in agreement with structural studies showing that these substitutions adversely affect interactions with FQs by modifying the geometric properties of the QBP and by displacing the conserved water molecules involved in Mg^{2+} ion preservation, which is essential for FQ activity (13). The functional analysis of other *F. novicida* mutated GyrA subunits revealed that the P43H substitution, which has not been reported before, also contributes to FQ resistance, though to a lesser extent. The capacity of mutations outside QRDR-A to confer small increases in the MICs of ciprofloxacin and gatifloxacin was previously described for *E. coli* (A51V mutation) (24). The molecular model of the structure of the functional core of *F. novicida* DNA gyrase presented here shows that residue P43, which is conserved in several pathogens (Fig. S2), is located in close proximity to the catalytic tyrosine residues and to DNA and may thus play a role in the interaction between FQs and their target. In contrast, deletions within the C terminus of GyrA (ΔE524 and ΔS525) are not associated with resistance to ciprofloxacin. DNA supercoiling assays also showed that GyrB mutations are associated with high-level FQ resistance in *F. novicida*. Thus, insertion of residue P466 into QRDR-B alters the FQ inhibitory effect on supercoiling as efficiently as the QRDR-A D87 substitution does. While it is inactive on its own, the D487E-ΔK488 mutation indirectly contributes to the acquisition of a high level of FQ resistance in double GyrA/GyrB mutants, as confirmed by complementation experiments carried out on mutated lineages of *F. novicida* resistant to FQs.

In Gram-negative bacteria, resistance to FQs has predominantly been attributed to GyrA mutations, with GyrB mutations being encountered less commonly and often found secondary to alterations in GyrA (14). In Gram-positive bacteria, depending on the FQs used, different levels of inhibitory activity against DNA gyrase and topoisomerase IV are observed, but in several cases topoisomerase IV seems to be the more sensitive enzyme (25). In this case, the C subunit of topoisomerase IV (ParC) is considered the primary target of FQs, whereas mutations affecting the E subunit (ParE) occur at a lower frequency and as second-step mutations.

Here we identified, for the first time, mutations in GyrB that are associated with a high level of resistance and that represent the first step in selection for decreased susceptibility to FQs. This unusual order, in which GyrB is altered before GyrA, is consistent with our data on topoisomerase IV. While in the *F. novicida* lineages examined here topoisomerase IV mutation is a rare event that occurs at the late stage of FQ exposure and seems to play a minor role in the development of FQ resistance, it should be noted that the ParE substitution observed in the Fno2 lineage is not preceded by ParC changes. Such an unusual first-step mutation in GyrB was confirmed following a single-step selection with ciprofloxacin on plates *in vitro* (Table S3). Thus, of the 15 independent single-step mutants selected, 5 had a QRDR-A mutation and 10 shared a GyrB substitution, including 1 isolate with a P466 insertion and 7 isolates with a substitution of the GyrB S465 residue. Interestingly, a first-step mutation in GyrB was also recently evidenced in an FQ-resistant *Salmonella enterica* isolate from an infected patient (26). From this observation, it can be hypothesized that the mutation pattern observed in *F. novicida* is not based on the *in vitro* selection protocol but is likely to be observed *in vivo*. The preponderant role of GyrB in FQ resistance highlighted here fits well with the recently proposed inverted binding mode of FQs to gyrase-DNA complexes (27). Using *Mycobacterium smegmatis* as a model, Mustaev et al. (27) indeed proposed a role for the GyrB E466 or K447 residue as an alternative way to ensure stabilization of the drug-enzyme-DNA complex known to be mediated through the magnesium-water bridge with GyrA residues 83 and 87.

Overall, our findings provide new insights into the molecular mechanisms of FQ resistance in *F. novicida*. We noted some discrepancy between the IC_{50} of reconstituted DNA gyrase and the MICs for resistant bacterial lineages, most particularly concerning Fno3 and Fno4 mutants, suggesting that other mechanisms, independent of GyrA or GyrB mutations, contribute to resistance, as is the case for several human pathogens (28, 29). Genome sequencing of the FQ-resistant *F. novicida* isolates identified, for each of the four lineages, a mutation within the promoter of the FTN_0480 gene, encoding the 110-amino-acid Fev protein, which is weakly homologous to the MerR family of transcription factors and characterized as an essential regulator of virulence in *Francisella* (30). Given that reports on the incidence of promoter mutations in *Francisella* are lacking, it is difficult to speculate on the consequences of this mutation for resistance to FQs. With this exception, the mutations detected are specific for each lineage. A set of three to five targets is mutated after antibiotic exposure, leading to several interrupted coding sequences. Concerning Fno3, for which the IC_{50} against DNA supercoiling is low compared to the bacterial MIC, nonsense mutations are present in the *pilB* and *pilE* genes, required for the expression of functional type IV pili (31), as well as in FTN_1029, coding for a protein involved in the isoprenoid biosynthesis pathway. Interestingly, this metabolic pathway, known to be involved in major cellular processes, including cell wall biosynthesis or the regulation of membrane fluidity (32), has been described as a promising target for the development of novel antimicrobial drugs against *F. tularensis* (33). Another nonsense mutation is found in Fno3 *wbtQ*, which contributes to the biosynthesis of the *F. novicida* O antigen involved in bacterial resistance to macrolides (34). The *wbtH* gene targeted in the Fno2 lineage belongs to the same gene cluster. Overall, we noted that several mutations target genes encoding proteins located at the membrane or involved in transmembrane transport that may be impaired in their functions, leading to changes in antibiotic susceptibility. The erythromycin and doxycycline cross-resistance patterns of *F. novicida* lineages highly resistant to FQs reported in the work of Sutera et al. (11) reinforce the hypothesis of alterations in outer membrane permeability. While the precise consequences of such mutations remain to be investigated, it is tempting to speculate that they are somehow relevant to the emergence of *F. novicida* mutants that are highly resistant to FQs. This hypothesis is reinforced by the data of Jaing et al., who identified proteins possibly involved in *F. tularensis* ciprofloxacin resistance through a careful analysis of both microarray and next-generation sequencing data for 11 isolates that had acquired resistance *in vitro* upon exposure to increasing antibiotic concentrations (19). Besides the GyrA and GyrB substitutions, three identified *F. tularensis* LVS mutated targets are also mutated in *F. novicida*, including *fupA* (FTL_0439/FTN_0444), coding for the protein responsible for siderophore-mediated iron acquisition and thought to be involved in bacterial virulence (35, 36); *wbtQ* (FTL_0601/FTN_0430), as discussed above; and an efflux pump-encoding gene (FTL-1547/FTN_1609).

In summary, while the whole-genome analyses of resistant clones suggest that the response to FQ exposure might be much more complex than initially thought, we have functionally characterized, for the first time, DNA gyrase mutations through which FQ resistance emerges in *F. novicida*.

MATERIALS AND METHODS

Sample collection. The phenotypic and genotypic characteristics of the *F. novicida* lineages selected for this study are reported in Table 1. In contrast to the FQ-susceptible parental strain *F. novicida* U112, the four lineages studied here, designated Fno1 to Fno4 and propagated for 14 passages in the presence of ciprofloxacin, are highly resistant to FQs (11). A common feature of these FQ-resistant strains is the presence of mutations in the gene encoding GyrA. Concerning Fno1 and Fno2, the GyrA substitutions are localized within the QRDR and are accompanied by additional GyrB mutations, whereas Fno3 and Fno4 are mutated in GyrA regions outside the QRDR, without an additional GyrB mutation. Bacterial cultures grown from glycerol stocks stored at −80°C at each passage were used for sequencing, MIC determination, and preparation of competent cells. Whole-genome sequencing was performed for each lineage (Fno1 to Fno4) on one clone at the end of the evolution experiment (passage 14), using ABI Solid sequencing technology (ABI 5500xl genetic analyzer), and mutations observed were further confirmed by PCR amplification and sequencing of the corresponding DNA regions by use of a dye terminator sequencing approach.

Expression and purification of *F. novicida* GyrA and GyrB. The genes for GyrA and GyrB were amplified from genomic DNAs of the selected isolates by use of gene-specific primers (see Table S1 in the supplemental material) and were cloned into the pDEST-17 vector with a 6×His N-terminal tag sequence (Invitrogen, Carlsbad, CA). The integrity of all constructs was confirmed by DNA sequencing (Eurofins, Ebersberg, Germany). Protein expression was carried out in *Escherichia coli* BL21(DE3) or C41(DE3) grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.6 in Luria broth (LB) supplemented with 100 μg/ml ampicillin before addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). Optimized conditions for each mutant are reported in Table S2. After sonication, the lysate was clarified by centrifugation and then passed over a Ni^{2+} affinity column (Qiagen) and extensively washed with Tris-HCl–NaCl buffer supplemented with 25 to 50 mM imidazole before elution of the His-tagged proteins in the presence of 300 mM imidazole. The purity of recombinant proteins was analyzed by SDS-PAGE and Coomassie blue staining before concentration using an Amicon Ultra centrifugal device. Purified proteins (0.5 to 1.0 mg/ml) were dialyzed overnight at 4°C (50 mM Tris-HCl, pH 7.9, 40% glycerol, 1 mM EDTA, 1 mM DTT) and stored at −80°C after flash freezing in liquid nitrogen. For Fno2 GyrA, 500 mM NaCl was added to the dialysis buffer to avoid protein precipitation.

DNA supercoiling assays. DNA supercoiling assays were carried out with reconstituted combinations of the GyrA and GyrB subunits from the wild-type and/or mutated strain. The protocol was similar to that applied for the analysis of *Mycobacterium tuberculosis* DNA gyrase (37), with minor modifications. The experiments were conducted in a final volume of 10 μl containing 35 mM Tris-HCl, pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM DTT, 0.1 mg/ml BSA, 1.8 mM spermidine, 100 mM potassium glutamate, 1 mM ATP, 6.5% (wt/vol) glycerol, and 0.15 μg relaxed pBR322 (Inspiralis, Norwich, United Kingdom) as the substrate. After 30 min of incubation at 37°C, the reaction was stopped by adding gel loading dye (New England BioLabs). Samples were analyzed by electrophoresis in 0.8% agarose gels run at 50 V for 6 h in 1× Tris-acetate-EDTA (TAE) buffer, stained with GelRed, and imaged on a UV transilluminator. For all combinations of GyrA and GyrB tested, we determined the minimal amounts of wild-type and/or mutated proteins required to convert 0.15 μg of relaxed pBR322 DNA to the supercoiled form (1 U). The inhibitory effect of ciprofloxacin (Sigma-Aldrich) or moxifloxacin (Bayer) against the wild-type and mutated DNA gyrases was assessed by determining the concentration of drug required to inhibit supercoiling activity by 50% (IC_{50}). Each experiment was conducted at least in triplicate, using two different batches of proteins. Statistical analysis was done using Prism v5.0 (GraphPad). The unpaired Student *t* test was used to determine the difference between the means of values obtained in independent experiments, with *P* values of <0.05 being considered significant.

DNA cleavage assays. DNA cleavage assays were performed in a 10-μl reaction volume, using the same buffer as that for supercoiling assays (but without ATP), 0.2 μg supercoiled pBR322 DNA (Inspiralis, Norwich, United Kingdom) as the substrate, and 125 nM (each) GyrA and GyrB subunits. Following 1 h of incubation at 25°C, SDS and proteinase K were added, and the incubation was continued for 30 min at 37°C as described previously (38). The extent of DNA cleavage was quantified after electrophoresis as described above, and the result was expressed as the FQ concentration required to induce 25% of the maximum DNA cleavage (CC_{25}).

Molecular modeling. The breakage reunion (GyrA N terminus) and Toprim (GyrB C terminus) domains were modeled using Phyre2 (39). The catalytic core model, composed of the breakage reunion and Toprim domains, DNA, and FQs, was generated by superpositioning onto the crystal structure of the *M. tuberculosis* DNA gyrase (40), using SSM implemented in Coot (41).

Transformation of *F. novicida* with a GyrB-encoding plasmid. The gene encoding *F. novicida* wild-type or D487E-ΔK488 GyrB was amplified using a primer pair in which NotI and AgeI restriction sites had been engineered (Table S1) and then was cloned into the pFNLTP6 shuttle plasmid downstream of the *gro* promoter (42) by use of T4 DNA ligase (New England BioLabs). Following restriction enzyme digestion and sequencing, the constructs were introduced into selected *F. novicida* isolates by electroporation as described previously (43). Transformed colonies appeared after 2 days of incubation, and the presence of the plasmid was checked by PCR using specific primers (Table S1).

Accession number(s). The sequences of the final evolved clones are available in the Sequence Read Archive under accession numbers SRX2188198, SRX2188197, SRX2188196, and SRX2188195.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02277-16.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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